Synergistic Anti-SARS-CoV-2 Activity of Repurposed Anti-Parasitic Drug Combinations

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Research Article

Keywords: SARS-CoV-2, repurposed drug, anti-parasitic drugs, Niclosamide, Ivermectin, Chloroquine

Posted Date: November 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1069947/v1

Abstract

COVID-19 pandemic has claimed millions of lives and devastated the health service system, livelihood and economy in many countries worldwide. Despite the initiation of vaccination programs in many countries, the spread of the pandemic continues and effective treatment is still urgently needed. Although some antiviral drugs have been shown to be effective, they are not widely available. Repurposing of anti-parasitic drugs with *in vitro* anti-SARS-CoV-2 activity is a promising approach being tested in many clinical trials. Combination of these drugs is a plausible way to enhance their effectiveness. We tested *in vitro* anti-SARS-CoV-2 activity of combinations of Niclosamide, Ivermectin, and Chloroquine; and show here that these combinations resulted in more than 10-fold reduction in the half maximal inhibitory concentration (IC_{50}) as compared to individual drugs. Synergy landscape analyses showed Niclosamide-Ivermectin combination to have the best synergy score with a peak Loewe synergy score of over 20 and a mean score of 6.60 in Vero E6 cell and a peak Loewe synergy score of 13.2 and a mean score of 2.897 in Calu-3 cells.

Introduction

The spread of SARS-CoV-2 and the COVID-19 pandemic has swept through countries and continents causing catastrophic loss to lives, public health, livelihood, and economy. Up to March 2021, more than hundred million cases have been reported with over two million deaths [2]. The hope to get through the pandemic and resume normal life relies heavily on vaccine deployment, which will still take months or years in most less-developed countries. One of the reasons for the heavy loss of lives, hospital overload, and public panic is the lack of effective treatment. Remdesivir is now the only antiviral drug with emergency use authorization by US FDA [39]. The drug is, however, not yet widely available. Other FDA-approved drugs are anti-inflammatory targeting host inflammatory responses [38]. More drugs capable of inhibiting SARS-CoV-2 replication are urgently needed not only for treatment but also for reducing viral load and transmission. Many repurposed anti-parasitic drugs have been shown to possess *in vitro* activity against SARS-CoV-2.

In vitro screenings of FDA-approved drugs have identified a number of anti-parasitic drugs with anti-SARS-CoV-2 activity and potential for drug repurposing for treatment of COVID-19 patients [4, 20]. The early hope to get an effective treatment using these drugs was let down by the failure to show clinical benefit of Chloroquine in clinical trials [35]. On the other hand, Ivermectin has shown promising results in many clinical trials [1, 6, 10–12, 17, 25, 40]. Ivermectin has been shown to cause up to 5000-fold reduction in SARS-CoV-2 replication *in vitro* [9, 13, 19]. The drug has been widely used to treat various parasitic diseases in humans and animals for four decades with little safety concern. It was also used in the mass treatment campaign against river blindness (Onchocerciasis) with good safety record [32]. It is therefore, an attractive option for drug repurposing for COVID-19 treatment. Another anti-parasitic drug, Niclosamide, showed a good anti-SARS-CoV-2 activity with a high selective index [20, 28]. The drug has been shown to exhibit broad antiviral activity against a wide range of viruses [45]. These anti-parasitic drugs with potent *in vitro* anti-SARS-CoV-2 activity are widely available, inexpensive, and considered

relatively safe for short-term usage. They were therefore selected for synergistic testing in order to find combination regimens with good potential for drug repurposing in COVID-19 treatment. The world urgently needs repurposed drug regimens with higher anti-SARS-CoV-2 activity in order to cope with the pandemic. An approach to enhance drug potency is through drug combination.

Materials And Methods

Chemicals

The 10 mM stock solutions were prepared in culture-grade 100% DMSO (Sigma) for Niclosamide (N3510, Sigma), and Ivermectin (I8898, Sigma), or water for Chloroquine (HY-17589, MCE) and stored at -80 ºC. All drugs were diluted to the working concentrations in 2%FBS-MEM or 2%FBS-DMEM/F12 for the treatments in Vero E6 and Calu-3 cells, respectively. The final concentration of DMSO was 0.5% in all experiments.

Cells And Viruses

Vero E6 (CRL-1586, ATCC) cells were cultivated in the minimum essential medium (MEM; 10-009-CV, Corning) supplemented with 10% heat inactivated FBS (10%FBS-MEM) at 37°C with 5% CO_2. Calu-3 cells (HTB-55, ATCC) were cultivated in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; 11320033, Gibco) supplemented with 10% heat inactivated FBS (10%FBS-DMEM/F12) at 37°C with 5% CO_2.

SARS-CoV-2 (SARS-CoV-2/01/human/Jan2020/Thailand) was previously isolated from nasopharyngeal swabs of a COVID-19 case in Thailand [22]. The virus was propagated in Vero E6 cells. The supernatants containing virus were harvested by centrifugation to remove cell debris, then aliquot and stored at -80°C. The viral titer was determined by plaque assay or 50% tissue culture infectious dose ($TCID_{50}$) endpoint dilution assay.

Virus Infection

Vero E6 or Calu-3 cells were seeded in culture plates at a density that allowed 100% and 70% confluence to be reach, respectively. The cell culture supernatants were removed and the cells were incubated with 2%FBS-MEM or 2%FBS-DMEM/F12 containing SARS-CoV-2 at the indicated multiplicity of infection (m.o.i.) or 2%FBS-media as a mock infection for one hour at 37°C with 5% CO2. Subsequently, the viral inoculum was removed, and the cells were maintained in 2%FBS-MEM or 2%FBS-DMEM/F12 for Vero E6 or Calu-3 cells, respectively, for the indicated time periods.

Viral Titration

Plaque assay

Vero E6 cells were plated in 24-well plates at a density of 1.3×10^5 cells per well before the day of infection. Then the culture medium was removed, and the cells were inoculated with 10-fold serial dilution of virus supernatants for one hour at 37^{o}C with 5%CO_2. Subsequently, the virus supernatants were removed, and the cells were overlaid with 1 ml of 1.56% microcrystalline cellulose (Avicel, RC-591) in 2%FBS-MEM. The cells were incubated at 37^{o}C with 5%CO_2 for three days. The overlaid medium was removed, and the cells were fixed with 10% (v/v) formalin in phosphate-buffered saline (PBS) for 2 hr. The fixed infected cells were washed in tap water and stained with 1% (w/v) crystal violet in 20% (v/v) ethanol for 5 min and washed to remove the excess dye. The plaques were counted and the viral titers were calculated in plaque forming units per ml (pfu/ml).

Tcid Endpoint Dilution Assay

Calu-3 cells were seeded in 96-well plates at a density of 2.5×10^4 cells/well. The culture medium was removed, and the cells were incubated with half-log10 serial dilution of the virus stock for 2 days at 37^{o}C with 5%CO_2. After that, the cells were fixed with 1:1 methanol/acetone for 30 min at 4^{o}C and the infectivity was detected with an antibody against the SARS-CoV-2 nucleocapsid protein (40143-R001, Sino Biological) and the appropriate secondary antibody-congugated HRP. The viral $TCID_{50}$ titers were calculated using the Reed and Muench method [31].

One-step Quantitative Reverse-transcription Pcr (Qrt-pcr)

The one-step qRT-PCR was used as a screening assay to detect the RNA of SARS-CoV-2 directly from the virus supernatants, without RNA purification [14]. Virus supernatants were heat inactivated at 70^{o}C for 20 min and diluted with DNase/RNase free distilled water for a ratio of 1:10. Subsequently, one-step qRT-PCR was performed using the Power SYBR one-step kit (Applied Biosystems) and the LightCycler 480 (Roche, LC480). The one-step RT-PCR master mix was prepared following the kit's instructions for a 10 µl reaction volume.

The primers used were CCDC-N-Fw: 5'-GGGGAACTTCTCCTGCTAGAAT-3'and CCDC-N-Rv: 5'-CAGACATTTTGCTCTCAAGCTG-3'. The master mix was mixed with 4.6 µl of the diluted sample in 96-well white PCR plate. RNA of SARS-CoV-2 purifying from the virus stock using TRIzol-LS (Invitrogen) was used as a positive control. The samples also include no-template control (nuclease-free water and the medium of mock infected cells). The LC480 was run according to the Power SYBR one-step kit's instructions. For briefly, the revere transcription step at 48^{o}C for 30 min and the activation of polymerase at 95^{o}C for 10 min. Then followed by 45 amplification cycles (95^{o}C for 15s, 60^{o}C for 1 min) and melt curve step to

determine the specificity of the PCR product from the melting temperature (T_m) (95^{o}C for 30s, 60^{o}C for 30s).

The threshold cycle (Ct) values were calculated from raw fluorescence data using Abs Quant/2nd derivative method. The T_m calling analysis was performed to exclude reactions with non-specific amplification by comparing with the product amplified from positive control and the no template control. The inhibition of SARS-CoV-2 production in drug-treated cells was relative to the cells treated with 0.5% DMSO-2%FBS-MEM.

Cell Viability Assay

Vero E6 or Calu-3 cells were seeded in 96 well-plates at a density that allowed 100% and 70% confluence to be reached, respectively. The culture medium was removed, then various concentrations of drugs in 2%FBS-MEM or 2%FBS-DMEM/F12 were added to the cells for 48 hours. After that, the cell viability was assessed using MTT dye (Invitrogen). The viable cells would convert the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide to MTT formazan. The precipitates of MTT formazan in the cells then were dissolved by DMSO. The absorbance was measured at 570 nm. The cells treated with 0.5% DMSO were used as a control (100% cell viability).

Evaluations of antiviral activity against SARS-CoV-2 in vitro

Single Drug Treatments

Vero E6 or Calu-3 cells were seeded in 96 well-plates at a density of 2.5x10^4 or 2.0x10^4 cells per well, respectively. The drugs were serially diluted by twofold in 2%FBS-media. Then the cells were incubated with the serial dilution drugs or no drug control (0.5% DMSO) for one hour at 37^{o}C with 5% CO_2. Subsequently, the virus at m.o.i. 0.01 or 500 $TCID_{50}$/100µl was added to Vero E6 or Calu-3 cells, respectively, and incubated for one hour. After that, the mixtures of drug and virus were removed and the cells were further maintained in the 2%FBS-media containing the serial dilution drugs or 0.5% DMSO for 48 hours. The culture supernatants were collected and the viral titers were determined using a plaque assay and one-step qRT-PCR.

Two-drug Combinations Treatments

Vero E6 or Calu-3 cells were seeded in 96 well-plates at a density of 2.5x10^4 or 2.0x10^4 cells per well, respectively. The cells were treated for one hour with 16 different pairwise combinations of two drugs. The drug concentrations ranged between 2×, 1×, 0.5× and 0.25× of IC_{50} values. Subsequently, the cells were infected with SARS-CoV-2 following the same approach used in single drug treatment. The virus supernatants were collected for titration by qRT-PCR or a plaque assay.

The Combination Synergy Analysis

The SynergyFinder web application was used to analyze and visualize the degree of combination synergy between two drugs. The synergy scores of two-drug combinations were analyzed by comparing the observed drug combination response (percent inhibition) against the expected response, calculated using a reference model [18]. Four reference models were used in this study, including the Loewe additivity (Loewe), Zero Independence Potency (ZIP), Highest Single Agent (HSA), and Bliss independence models [26].

Statistical analysis

The independence experiments were performed in triplicated, and data are shown as mean ± SD. The 50% cytotoxic concentration (CC_{50}) and the half-maximal inhibitory concentration (IC_{50}) were calculated from the dose-response curves of drug treatment against SARS-CoV-2 by non-linear regression analysis using GraphPad Prism 8 (GraphPad Software, Inc., CA).

Results

Evaluation of single drug treatment against SARS-CoV-2 in Vero E6 cells

Figure 1 and Table 1 show the anti-SARS-CoV-2 activities and cytotoxicity of the repurposed drugs in Vero E6 cells. The plaque assay was used to determine the viral production and is expressed as the percent inhibition relative to the viral titer of DMSO-treated cells. The one-step qRT-PCR was used to quantitate the viral RNA in virus supernatants and is also expressed as the percent inhibition relative to the DMSO-treated cells. The IC_{50} values calculated from the dose-response determined by plaque assay for Niclosamide, Ivermectin, and Chloroquine were 0.049, 1.23, 0.046 and 0.83 μM, respectively. The IC_{50} values calculated from the dose-response determined by one-step qRT-PCR for Niclosamide, Ivermectin, and Chloroquine were 0.043, 1.27, and 0.89 μM, respectively. Both methods used for viral quantification resulted in similar IC_{50} values. Thus the viral RNA quantification by the one-step qRT-PCR accurately determined the infectious virus output in these experiments, and could be used for the further two-drug combination experiments for the high throughput screening.

Table 1
Single drug treatment against SARS-CoV-2 *in vitro*.

Drug candidates	Drug class	Drug indication	CC_{50} (μM)	IC_{50} (μM) Plaque assay	IC_{50} (μM) qRT-PCR
Niclosamide	Anthelminthic agents	Treatment of tapeworm and intestinal fluke infections [13]	0.29	0.049	0.043
Ivermectin	Anti-parasitic agents	Treatment of onchocerciasis, and other worm infestations [14]	10.55	1.23	1.27
Chloroquine	Anti-malarial agents	Treatment of malaria, rheumatic diseases and Zika virus infection [50]	118.20	0.83	0.89

Evaluation of two-drug combination treatments against SARS-CoV-2 in Vero E6 cells

Firstly, the antiviral activities of two-drug combinations were assessed *in vitro* in Vero E6 cells. The cells were treated with 16 different pairwise combinations of two drugs, including, Niclosamide-Ivermectin, Niclosamide-Chloroquine and Ivermectin-chloroquine.

Niclosamide-ivermectin Combination

The presence of Ivermectin induced a shift in the dose-response curve of Niclosamide, with 10.75-fold reduction of Niclosamide IC_{50} value in the presence of 2.4 and 1.2 μM Ivermectin and approximately 2-fold reduction of Niclosamide IC_{50} value in the presence of 0.6 and 0.3 μM Ivermectin (Fig. 2A, Table 2). In a similar way, the presence of Niclosamide also induced a shift in Ivermectin dose-response curve, with 26.46-fold reduction of Ivermectin IC_{50} value in the presence of 0.09 μM Niclosamide (Fig. 2B, Table 2). The presence of 0.045 μM, 0.0225 μM and 0.01125 μM Niclosamide resulted in 7.18, 4.06 and 1.92-fold reduction of Ivermectin IC_{50} value, respectively. The dose-response matrix of Niclosamide and Ivermectin combination showed the obvious increasing inhibitory effects (Fig. 2C). A synergy landscape plot showed high positive Loewe synergy scores in combinations with Ivermectin concentration higher than 0.6 μM with a peak score of 22.76 indicating a synergistic effect. The scores were low positive to slightly negative in the other part of the plot with lower Ivermectin concentration indicating only additive effect at these low concentrations (Fig. 2D). The mean Loewe synergy score is 6.60. The ZIP, Bliss independence and HSA reference models were also used, the results showed the synergy scores of 12.64, 12.77 and 19.03, respectively, which accounted for the synergistic effect between Niclosamide and Ivermectin in Vero E6. No significant cytotoxicity in all 16 pairwise combinations (Fig. 2A, B).

Table 2
Antiviral activity of two-drug combinations treatment against SARS-CoV-2 in Vero E6 cells.

Drug treatment		IC_{50} (μM) qRT-PCR	Fold reduction of IC_{50} (single/combined)
Niclosamide-Ivermectin	**Niclosamide**	**0.043**	
	Niclosamide + Ivermectin 2.4 μM	0.004	10.750
	Niclosamide + Ivermectin 1.2 μM	0.004	10.750
	Niclosamide + Ivermectin 0.6 μM	0.018	2.399
	Niclosamide + Ivermectin 0.3 μM	0.022	1.955
	Ivermectin	**1.27**	
	Ivermectin + Niclosamide 0.09 μM	0.048	26.46
	Ivermectin + Niclosamide 0.0045 μM	0.177	7.18
	Ivermectin + Niclosamide 0.0225 μM	0.313	4.06
	Ivermectin + Niclosamide 0.01125 μM	0.660	1.92
Niclosamide-Chloroquine	**Niclosamide**	**0.043**	
	Niclosamide + Chloroquine 1.7 μM	0.003	14.333
	Niclosamide + Chloroquine 0.85 μM	0.009	4.778
	Niclosamide + Chloroquine 0.425 μM	0.013	3.308
	Niclosamide + Chloroquine 0.2125 μM	0.029	1.483
	Chloroquine	**0.89**	
	Chloroquine + Niclosamide 0.09 μM	0.028	31.78

Drug treatment		IC_{50} (µM) qRT-PCR	Fold reduction of IC_{50} (single/combined)
	Chloroquine + Niclosamide 0.0045 µM	0.193	4.61
	Chloroquine + Niclosamide 0.0225 µM	0.249	3.57
	Chloroquine + Niclosamide 0.01125 µM	0.531	1.68
Ivermectin-Chloroquine	**Ivermectin**	**1.27**	
	Ivermectin + Chloroquine 1.7 µM	0.023	55.22
	Ivermectin + Chloroquine 0.85 µM	0.122	10.41
	Ivermectin + Chloroquine 0.425 µM	0.515	2.47
	Ivermectin + Chloroquine 0.2125 µM	0.821	1.55
	Chloroquine	**0.89**	
	Chloroquine + Ivermectin 2.4 µM	0.014	63.57
	Chloroquine + Ivermectin 1.2 µM	0.221	4.03
	Chloroquine + Ivermectin 0.6 µM	0.315	2.83
	Chloroquine + Ivermectin 0.3 µM	0.514	1.73

Niclosamide-chloroquine Combination

By using the same approach, it was found that the presence of Chloroquine induced a shift in Niclosamide dose-response curve, with 14.333, 4.778, 3.308 and 1.483-fold reduction of Niclosamide IC_{50} value in the presence of 1.7, 0.85, 0.425, and 0.2125 µM Chloroquine, respectively (Fig. 3A, Table 2). A similar trend was observed for the Chloroquine dose-response curve in the presence of Niclosamide, with 31.78, 4.61, 3.57 and 1.68-fold reduction of Chloroquine IC_{50} value in the presence of 0.09, 0.045, 0.0225 and 0.01125 µM Niclosamide, respectively (Fig. 3B, Table 2). The dose-response matrix shows increasing

inhibitory effect of the combination with higher concentrations of Niclosamide and Chloroquine (Fig. 3C). The synergy map shows positive synergy scores at high concentrations of both drugs, while the lower concentrations gave zero and negative synergy scores with a peak positive score of 18.57, indicating a synergistic effect. (Fig. 3D). As most parts of the surface had Loewe synergy scores between -10 and 10, except for the highest concentrations of both drugs, with a mean score of 0.073, it suggests an additive effect between Niclosamide and Chloroquine. Additionally, the synergy scores calculated using ZIP and Bliss independence reference models gave the values of 3.86 and 3.67, respectively, which similarly indicated the additive effect. The HSA model resulted in the synergy score of 11.41, which accounted for the small level in synergistic effect. No significant cytotoxicity in all 16 pairwise combinations (Fig. 3A, B).

Ivermectin-chloroquine Combination

The results showed that the presence of Chloroquine induced a shift in Ivermectin dose-response curve, with 55.22, 10.41, 2.47, 1.55-fold reduction of Ivermectin IC_{50} value in the presence of 1.7, 0.85, 0.425, and 0.2125 µM Chloroquine, respectively (Fig. 4A, Table 2). Similarly, the presence of Ivermectin also induced a shift in Chloroquine dose-response curve, with 63.57, 4.03, 2.83 and 1.73-fold reduction of Chloroquine IC_{50} value in the presence of 2.4, 1.2, 0.6 and 0.3 µM Ivermectin, respectively (Fig. 4B, Table 2). The dose-response matrix shows increasing inhibitory effect with higher concentrations of Ivermectin and Chloroquine (Fig. 4C). Most parts of the synergy surface show negative synergy scores except for a small positive area with a peak positive score of 7.43 at the highest concentration of Chloroquine (Fig. 4D). The peak negative score of the surface is -8.16. As all of the surface had Loewe synergy scores between -10 and 10 with a mean score of -1.812, it suggests an additive effect between Ivermectin and Chloroquine. Moreover, both ZIP, Bliss independence and HSA reference models showed the synergy scores of 1.97, 1.98 and 9.63 which indicated the additive effect. No significant cytotoxicity in all 16 pairwise combinations (Fig. 4A, B).

Evaluation of single drug treatment against SARS-CoV-2 in Calu-3 cells

The best antiviral activity and calculated synergy scores demonstrated in the treatment with Niclosamide-Ivermectin combination in Vero E6 cells. Therefore, this two-drug combination was selected for the further evaluation in the human lung cancer cell line, Calu-3. The antiviral activities of single Niclosamide and Ivermectin treatments were assessed in Calu-3 cells (Fig. 5). The IC_{50} values of both drugs were 0.2 µM in Calu-3 cells. The CC_{50} values of Niclosamide and Ivermectin were 5.62 µM and 3.10 µM, respectively. The SI values of Niclosamide and Ivermectin were 28.1 and 15.5, respectively.

Evaluation of Niclosamide-Ivermectin combination treatment against SARS-CoV-2 in Calu-3 cells

The strong shifts were observed in the dose-response curves of Niclosamide combined with 0.4 and 0.2 µM Ivermectin (Fig. 6A, Table 3). The pairwise combinations of four different concentrations of Niclosamide with 0.4 and 0.2 µM Ivermectin resulted in a similar percent inhibition, thus, it was unable to

calculate accurate IC_{50} values with the least curve fit. The presence of 0.1 and 0.05 μM Ivermectin also induced a shift in the dose-response curve of Niclosamide, in a similar level of 2.38 and 2.33-fold reduction of Niclosamide IC_{50} values, respectively (Fig. 6A, Table 3). In a similar way, the presence of Niclosamide induced a shift in Ivermectin dose-response curve with 8.69, 4.88, 3.64, and 2.41-fold reduction of Ivermectin IC_{50} value in the presence of 0.4, 0.2, 0.1 and 0.05 μM Niclosamide, respectively (Fig. 6B, Table 3). The dose–response matrix shows the increasing antiviral activity compared to the single drug treatments (Fig. 6C). The combination synergy analysis showed the mean Loewe synergy score of 2.897, which accounted for the additive effect between Niclosamide and Ivermectin in Calu-3 cells (Fig. 6D). Additionally, the peak Loewe synergy score was 13.19. The synergy score obtained from ZIP and Bliss independence reference models were 0.886 and 0.954, respectively, which also accounted for the additive effect. The synergy score calculated using HSA models was 10.795, which indicated a small synergistic effect between Niclosamide and Ivermectin. All 16 pairwise combinations showed no significant cytotoxicity (Fig. 6A, B)

Table 3
Evaluation of Niclosamide-Ivermectin combination treatments against SARS-CoV-2 in Calu-3 cells

Drug treatment		**IC_{50} (μM)**	**Fold reduction of IC_{50} (single/combined)**
Favipiravir-Ivermectin	**Favipiravir**	**0.20**	
	Niclosamide + Ivermectin 0.4 μM	ND	ND
	Niclosamide + Ivermectin 0.2 μM	ND	ND
	Niclosamide + Ivermectin 0.1 μM	0.084	2.38
	Niclosamide + Ivermectin 0.05 μM	0.086	2.33
	Ivermectin	**0.20**	
	Ivermectin + Niclosamide 0.4 μM	0.023	8.69
	Ivermectin + Niclosamide 0.2 μM	0.041	4.88
	Ivermectin + Niclosamide 0.1 μM	0.055	3.64
	Ivermectin + Niclosamide 0.05 μM	0.083	2.41

ND = not determined, cannot calculate IC_{50} with the least curve fit of the data sets.

Discussion

Our study shows that the repurposed anti-parasitic drugs, Niclosamide, Ivermectin and Chloroquine possess high *in vitro* activity against SARS-CoV-2 as the IC_{50} values are in the low micromolar range. These results on the IC_{50} against SARS-CoV-2 of these single drugs are in agreement with previous studies [9, 20, 28, 30].

Previous *in vitro* studies suggested that Ivermectin inhibits host importin alpha/beta-1 nuclear transport proteins, thus preventing the viruses from suppressing the host antiviral response [47]. Recently, it was found that Ivermectin may interfere with the attachment of SARS-CoV-2 spike protein to the ACE2 receptor on human cell membrane [23]. Several studies also reported antiviral activity of Ivermectin on other viruses such as Zika virus [5], Dengue virus [42] and Human immunodeficiency virus type 1(HIV-1) [41]. And with its board spectrum antiviral activity, Ivermectin is thought to act on host cells for its antiviral activity.

Niclosamide showed broad antiviral activity against a wide range of viruses such as SARS-CoV [43–45], MERS-CoV [15], Zika virus [46], HCV [36], Ebola virus [27] and HIV-1 [29]. Several evidences found in other viruses suggested the plausible mechanisms of Niclosamide in SARS-CoV-2 inhibition by blocking of viral entry via altering endosomal pH and the prevention of autophagy that lead to the inhibition of virus replication [15, 21, 37]. Although Niclosamide was originally thought to act on parasitic worms in the gut lumen and is barely absorbed to the blood stream, it was tested for various systemic repurposed treatments, and a maximal plasma concentration ranged from 35.7 to 182 ng ml^{-1} (corresponding to 0.11-0.56 μM) was observed in a pharmacokinetic study [3, 8, 24, 34]. This level exceeds the *in vitro* Niclosamide IC_{50} against SARS-CoV-2, especially when used in the tested combinations.

Chloroquine inhibits a broad range of viruses by blocking viral entry via inhibition of endosomal acidification [33]. It was recently shown that Chloroquine could not inhibit SARS-CoV-2 in human lung cells because of the expression of TMPRSS2 [16]. This may at least partially explain the lack of clinical efficacy of this drug. Despite these *in vitro* anti-SARS-CoV-2 activities, clinical application of these drugs to COVID-19 treatment has not yet been successful. While some clinical trials of Ivermectin on COVID-19 treatment have shown promising results [1, 6, 11, 12, 17, 25, 40], clinical trials for Chloroquine mostly showed negative results [35] and there have been little clinical data on Niclosamide. The lack of obvious clinical efficacy suggests that either these *in vitro* activities could not take effect *in vivo* or the activities may not be sufficiently potent. An obvious strategy to enhance the potency is drug combination. While combining direct acting antivirals with different targets almost always results in additive or synergistic effect, combining drugs that act on host machineries does not always cause a synergistic effect and can even result in an antagonistic effect [7, 30]. Selecting proper drug combinations with synergistic effect is therefore crucial for development of efficacious regimens. Our data may be useful in guiding the design of clinical trials that may generate a badly needed efficacious regimen for COVID-19 treatment and prevention.

In conclusion, our study demonstrated the benefit of combining Ivermectin, Niclosamide and Chloroquine on their anti-SAR-CoV-2 activities. Among the combinations, Ivermectin and Niclosamide showed the best synergistic profile. This combination should be further tested in clinical trials.

Declarations

Acknowledgement

This study was supported by the Chair Professor Program (P-20-52262), The National Science and Technology Development Agency (NSTDA), Thailand. This study was also partly supported by Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

Author Contributions

KJ (Methodology, Formal Analysis, Investigation, Writing – Original Draft Preparation), CB (Methodology, Formal Analysis, Investigation), SM (Methodology, Investigation), NP (Methodology, Investigation), SB (Resource), AT (Validation, Methodology), PA (Validation, Methodology), P. Auewarakul (Conceptualization, Supervision, Funding, Writing – Review and Editing). All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Biosafety

This study was approved by Mahidol University Biosafety Committee (approval no. MU 2020-008).

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Figures

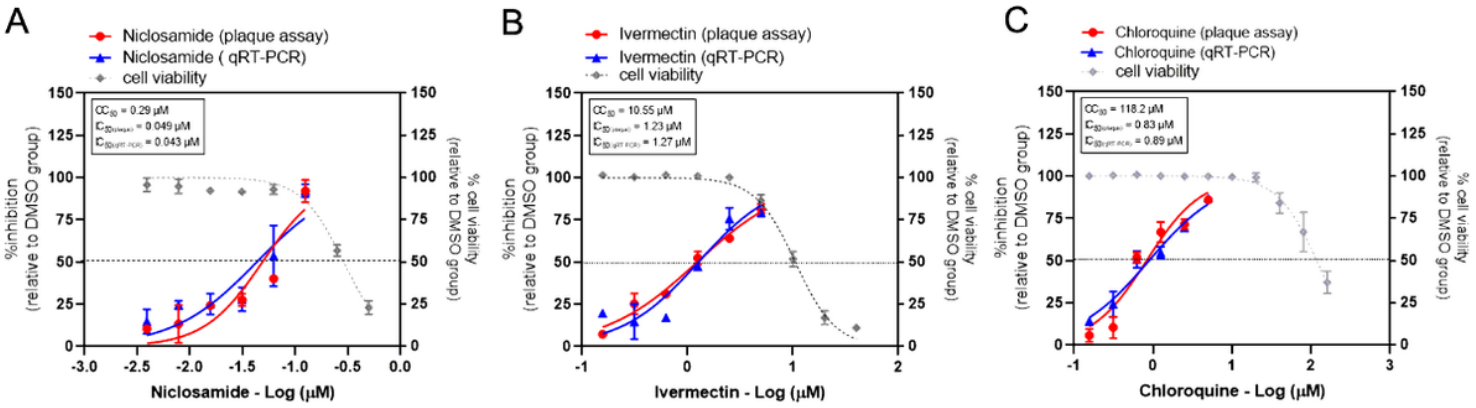

Figure 1

Evaluation of antiviral activity of drug candidates against SARS-CoV-2 in vitro The dose-response curves of a single drug treatment against SARS-CoV-2 were shown; (A) Niclosamide, (B) Ivermectin, and (C)

Chloroquine. Vero E6 cells were treated with twofold serial dilutions of drug for one hour and infected with SARS-CoV-2 at m.o.i. of 0.01. After removing of viruses, the cells were maintained in the medium containing serial dilutions of the single drugs or 0.5%DMSO for two days. The virus supernatants were collected for titration using the plaque assay and one step-qRT-PCR. The dose-response curves were expressed as the percent inhibition in relative to the infected-DMSO-treated cell control. The effect of drug treatment on the cell viability was determined using MTT assay and is expressed in relative to the DMSO-treated cell control. The experiments were repeated at least three times, and data are shown as mean ± SD.

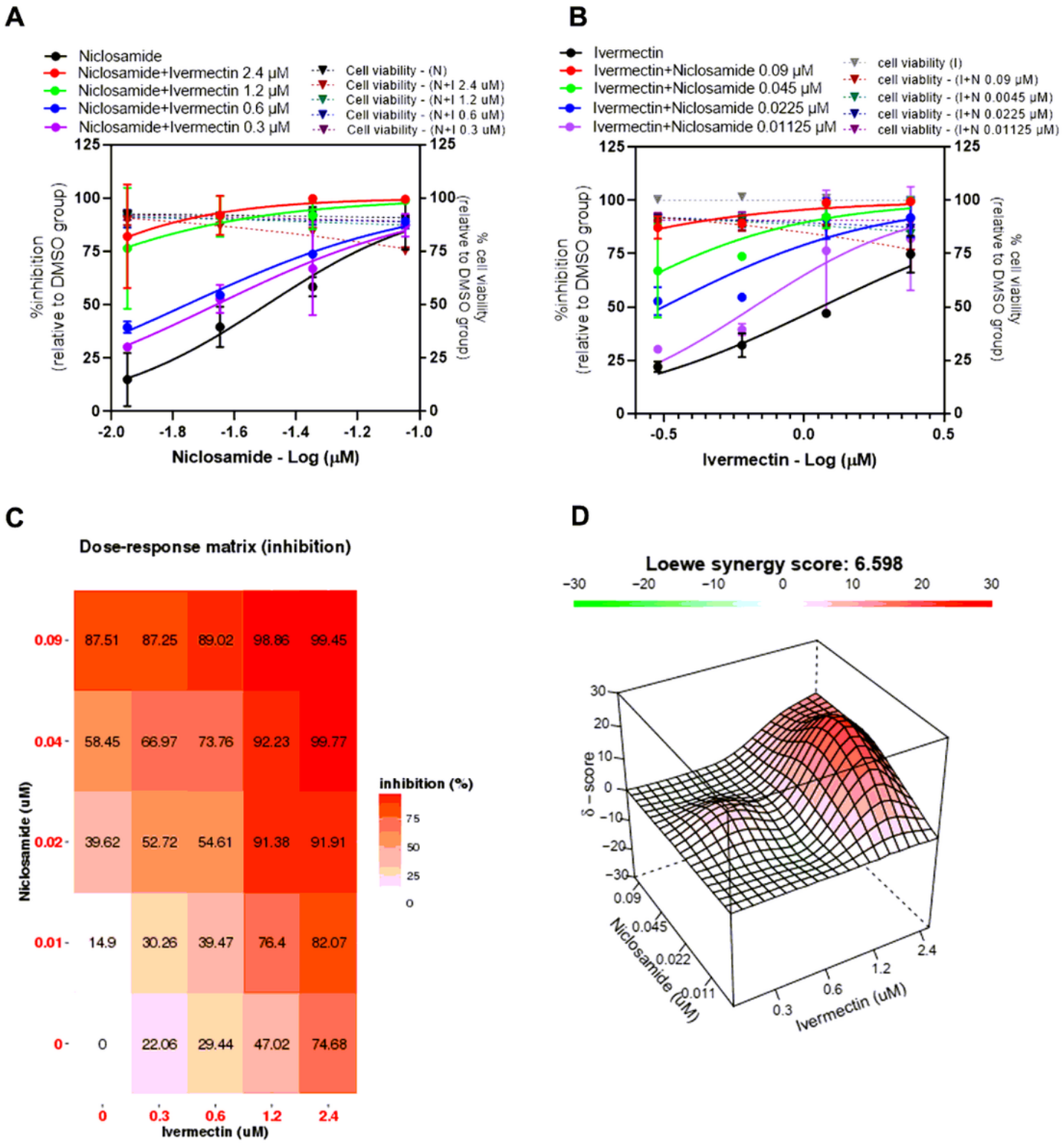

Figure 2

2Niclosamide-Ivermectin combination treatments against SARS-CoV-2 in Vero E6 cells. Vero E6 cells were treated for one hour with twofold serial dilutions of Niclosamide in the presence of different fixed concentrations of Ivermectin (A) or alternatively, serial dilutions of Ivermectin in the presence of different fixed concentrations of Niclosamide (B). Then the cells were infected with SARS-CoV-2 at m.o.i. 0.01. After removing the virus inoculum, the cells were further maintained in the medium containing drugs for 2

days. The viral RNA was determined using one-step qRT-PCR. The SynergyFinder was used to calculate the synergy score of two-drug combinations from different 16 pairwise combinations. The dose-response matrix (C) and the synergy map of two-drug combinations treatment (D) were shown. The interaction landscape between two drugs was calculated using Loewe model. Areas with synergy score less than -10: the interaction between two drugs is likely to be antagonistic; from -10 to 10: the interaction between two drugs is likely to be additive; larger than 10: the interaction between two drugs is likely to be synergistic. The experiments were repeated at least three times, and data are shown as mean ± SD.

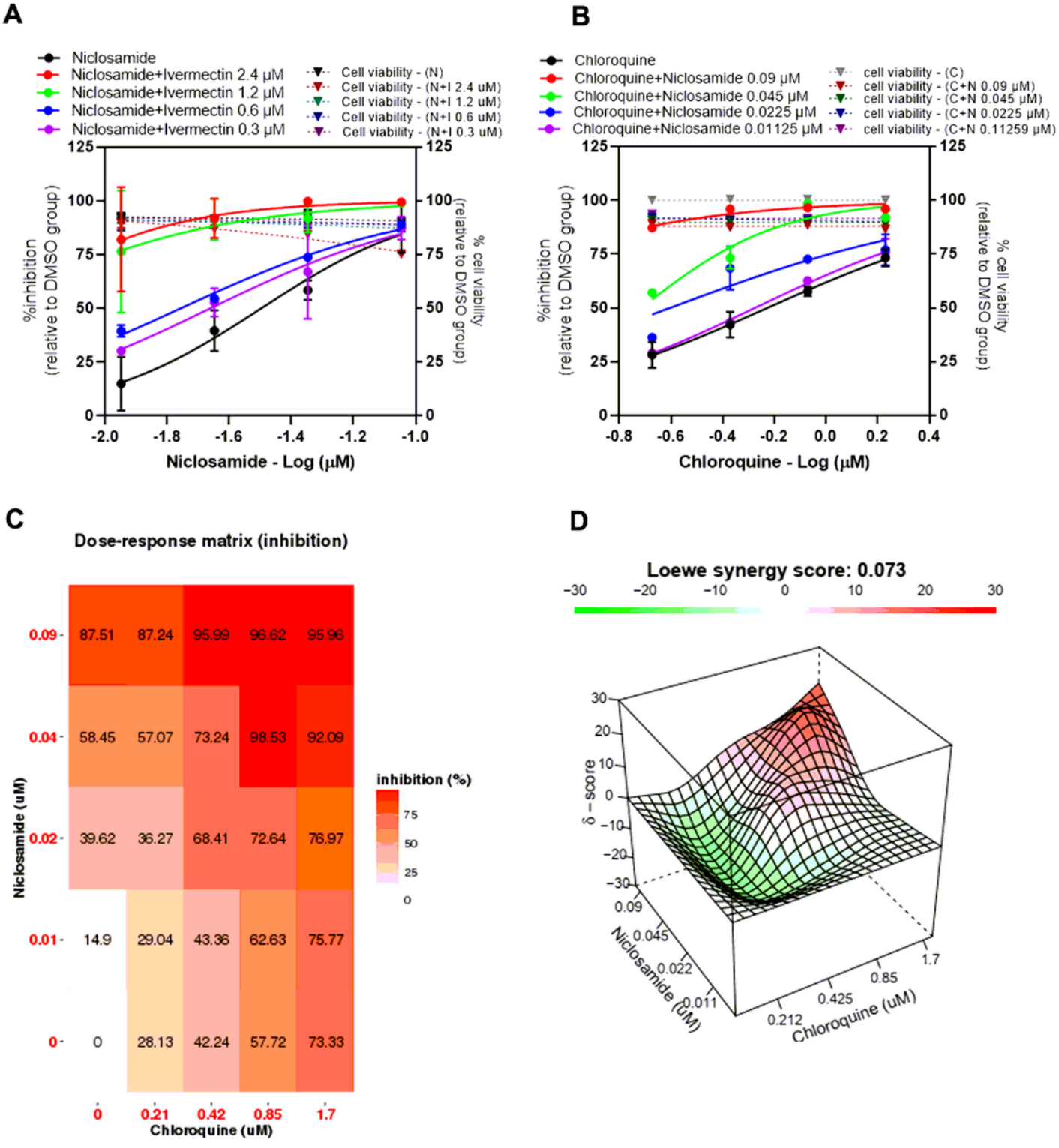

Figure 3

3Niclosamide-Chloroquine combination treatments against SARS-CoV-2 in Vero E6 cells. Vero E6 cells were treated for one hour with twofold serial dilutions of Niclosamide in the presence of different fixed concentrations of Chloroquine (A) or alternatively, serial dilutions of Chloroquine in the presence of different fixed concentrations of Niclosamide (B). Then the cells were infected with SARS-CoV-2 at m.o.i. 0.01. After removing the virus inoculum, the cells were further maintained in the medium containing drugs for 2 days. The viral RNA was determined using one-step qRT-PCR. The SynergyFinder was used to calculate the synergy score of two-drug combinations from different 16 pairwise combinations. The dose-response matrix (C) and the synergy map of two-drug combinations treatment (D) were shown. The interaction landscape between two drugs was calculated using Loewe model. Areas with synergy score less than -10: the interaction between two drugs is likely to be antagonistic; from -10 to 10: the interaction between two drugs is likely to be additive; larger than 10: the interaction between two drugs is likely to be synergistic. The experiments were repeated at least three times, and data are shown as mean ± SD.

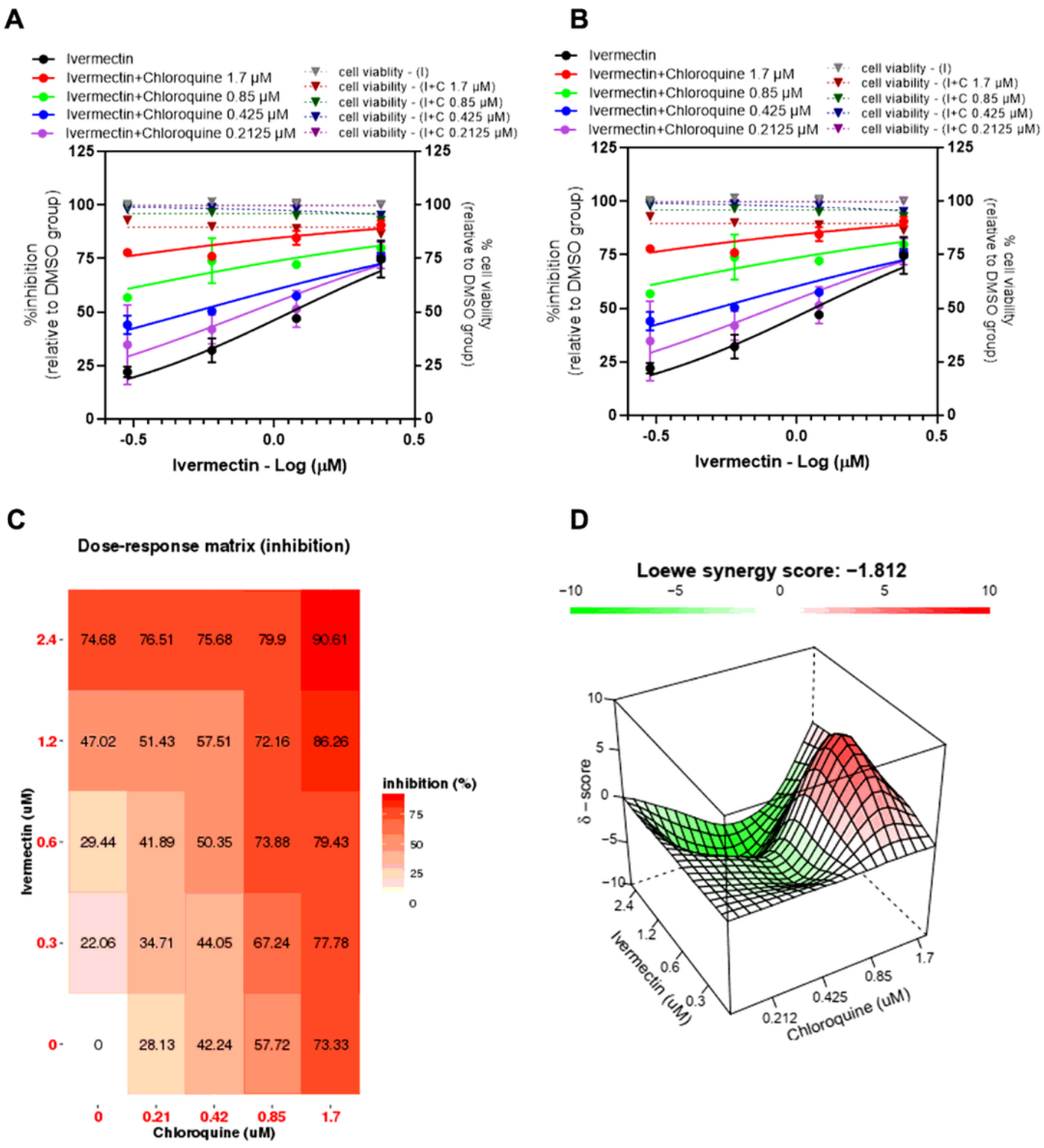

Figure 4

Ivermectin-Chloroquine combination treatments against SARS-CoV-2 in Vero E6 cells. Vero E6 cells were treated for one hour with twofold serial dilutions of Ivermectin in the presence of different fixed concentrations of Chloroquine (A) or alternatively, serial dilutions of Chloroquine in the presence of different fixed concentrations of Ivermectin (B). Then the cells were infected with SARS-CoV-2 at m.o.i. 0.01. After removing the virus inoculum, the cells were further maintained in the medium containing drugs

for 2 days. The viral RNA was determined using one-step qRT-PCR. The SynergyFinder was used to calculate the synergy score of two-drug combinations from different 16 pairwise combinations. The dose-response matrix (C) and the synergy map of two-drug combinations treatment (D) were shown. The interaction landscape between two drugs was calculated using Loewe model. Areas with synergy score less than -10: the interaction between two drugs is likely to be antagonistic; from -10 to 10: the interaction between two drugs is likely to be additive; larger than 10: the interaction between two drugs is likely to be synergistic. The experiments were repeated at least three times, and data are shown as mean ± SD.

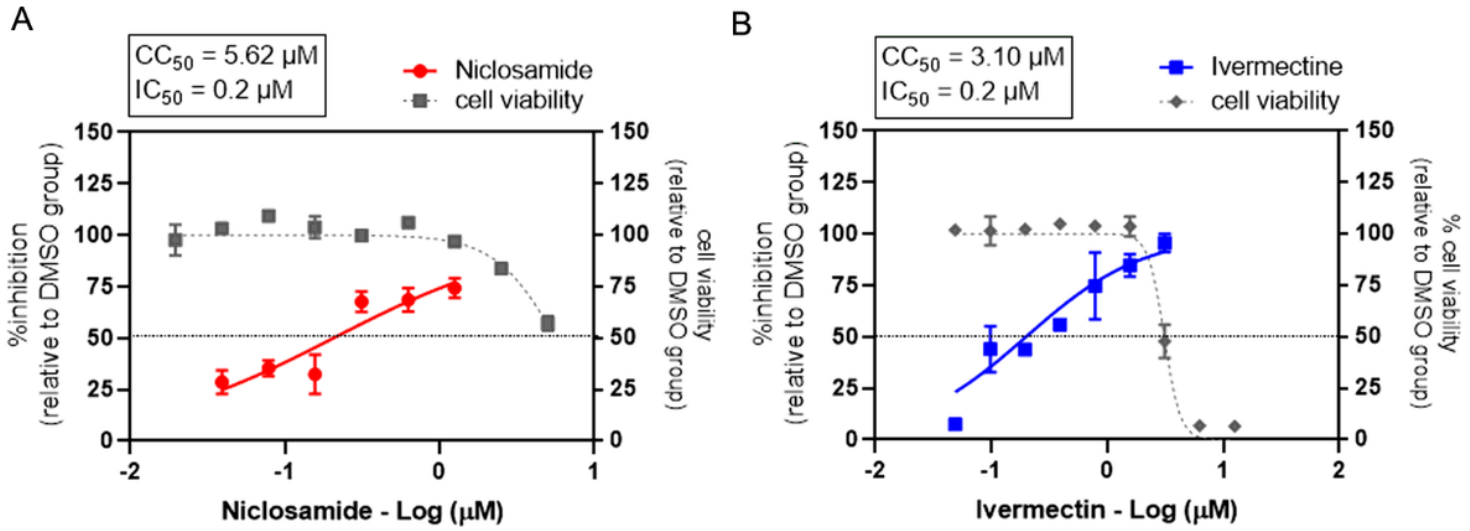

Figure 5

Single drug treatment against SARS-CoV-2 in Calu-3 cells The dose-response curves of a single drug treatment against SARS-CoV-2 are shown; (A) Niclosamide, and (B) Ivermectin. Calu-3 cells were treated with twofold serial dilutions of drug for one hour and infected with SARS-CoV-2. The infected cells were maintained in the medium containing serial dilutions of drugs or 0.5%DMSO for two days. Virus production was determined using a plaque assay. The dose-response curves are expressed as the percent inhibitions in relative to the DMSO-treated cell. The effect of drug treatment on cell viability was determined using MTT assay.

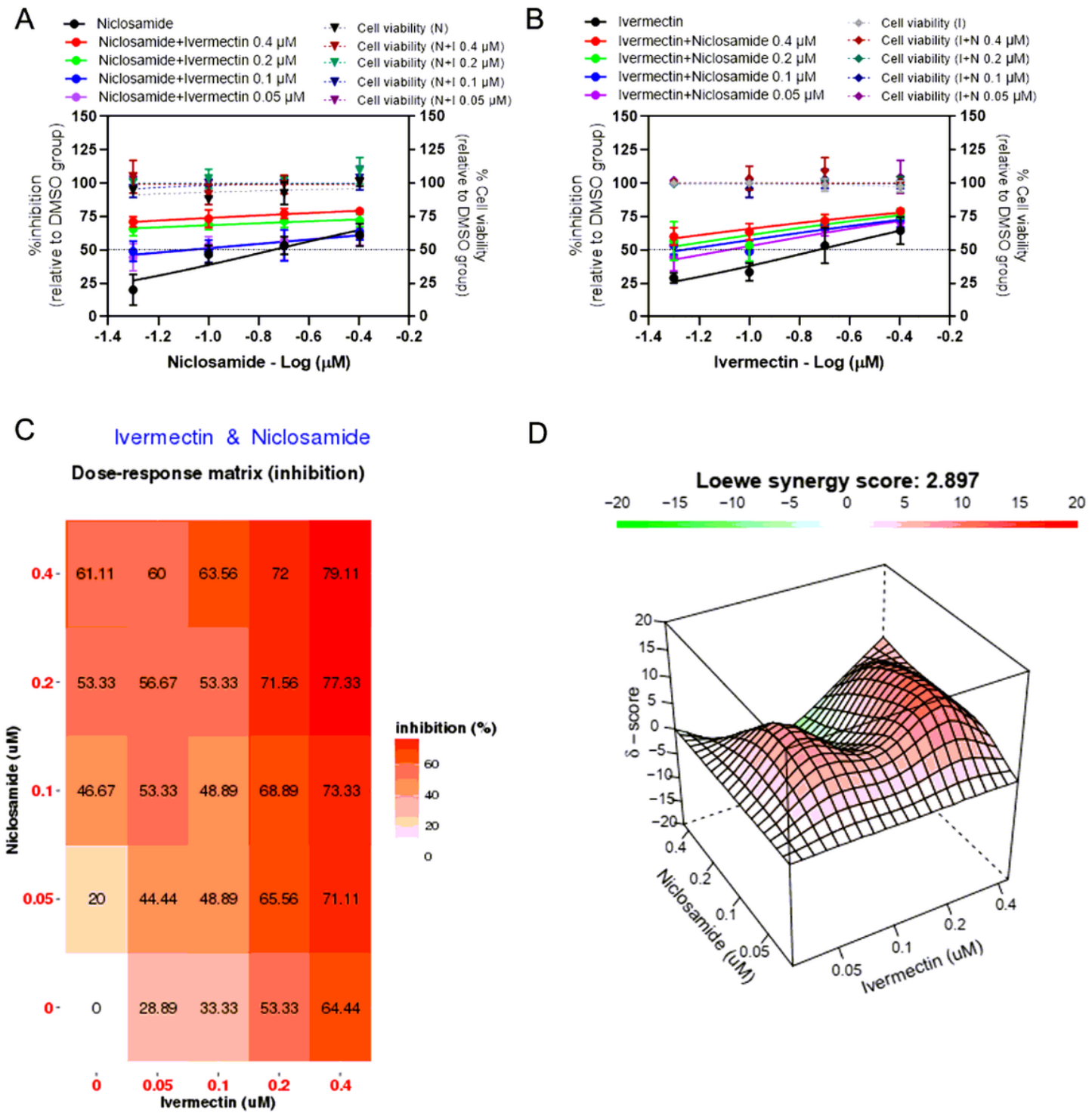

Figure 6

Niclosamide-Ivermectin combination treatments against SARS-CoV-2 in Calu-3 cells Calu-3 cells were treated for one hour with twofold serial dilutions of Niclosamide in the presence of different fixed concentrations of Ivermectin (A) or alternatively, serial dilutions of Ivermectin in the presence of different fixed concentrations of Niclosamide (B). Then the cells were infected with SARS-CoV-2 at m.o.i. 0.01. After removing the virus inoculum, the cells were further maintained in the medium containing drugs for 2 days. The viral RNA was determined using one-step qRT-PCR. The SynergyFinder was used to calculate

the synergy score of two-drug combinations from different 16 pairwise combinations. The dose-response matrix (C) and the synergy map of two-drug combinations treatment (D) were shown. The interaction landscape between two drugs was calculated using Loewe model. Areas with synergy score less than -10: the interaction between two drugs is likely to be antagonistic; from -10 to 10: the interaction between two drugs is likely to be additive; larger than 10: the interaction between two drugs is likely to be synergistic. The experiments were repeated for three times, and data are shown as mean ± SD.